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Segmented poly(ether–ester–amide)s based on poly(L,L-lactide) macromers

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Abstract

Segmented poly(ether–ester–amide)s (PEEAs) were obtained from sebacoyl chloride (SEB), α,ω-hydroxyl terminated poly(L,L-lactide) (PLLA) or PLLA–PEG–PLLA macromers and the hydrophilic diamines 4,7,10-trioxa-1,13-tridecanediamine (**1**) and 3,6,9,12,15-pentoxa-1,17-heptadecanediamine (**2**) or diamide–diamines derived from **1** and **2** by inserting Gly-Phe or Gly-Val-Phe sequences. The molar ratio macromer/SEB/diamine was 1/2/1. The PEEAs were characterized by i.r. and ^{1}H n.m.r spectroscopy and S.E.C. They are soluble in polar organic solvents and insoluble in water, but show equilibrium water absorptions up to 21.0% by wt. All PEEAs are semicrystalline, as shown by d.s.c. and WAXS techniques, with T_ms ranging from 110 to 135°C. D.s.c. and d.m.t.a. showed a single T_g in the temperature range 31–53°C. Evidence of microphase separation was obtained only for aminoacids containing PEEAs by t.e.m.. Biocompatibily was assessed for selected polymers checking the viability and growth of Caco-2 cells on polymer films.

Keywords: Block copolymers; Poly(ether–ester–amide)s; Biocompatible

1. Introduction

In recent years, efforts have been directed towards the preparation of biocompatible, biodegradable, reabsorbable condensation polymers with controlled lifetimes, which can be used to manufacture temporary implants or in controlled drug release [1,2]. Poly(L-lactic acid) (PLLA), poly(glycolic acid) and their copolymers have been intensively used in clinical applications, including tissue reconstruction and bioreabsorbable sutures, as they undergo hydrolytic degradation to non-toxic substances. However, they lack the physical and mechanical properties for a wider use. Aliphatic polyamides, on the other hand, are characterized by thermal stability and favourable mechanical properties, but they are generally considered non-degradable [3–5]. If α-aminoacids, however, are incorporated, they become potentially biodegradable [6–10]. Poly(ester–amide)s are an emerging class of biodegradable condensation copolymers, which combine the mechanical properties of polyamides and the biodegradability of the polyesters. They can be suitably tailored to the needs of their applications by varying the nature of the monomers and/or their composition and architecture. Polydepsipeptides, which are alternating copolymers derived from α-aminoacids and α-hydroxyacids [11,12], and block poly(ester-amide)s based on PLLA [13] belong to this class. An enhancement of biodegradability can be achieved by incorporating into the chain flexible, hydrophilic oligo(ethylenglycol) segments and α-aminoacids able to build up peptide linkages degradable through targeted cleavage by specific enzymes. Enzymatic degradation, however, depends on the chemical structure, crystallinity degree, chain flexibility and a balance of hydrophilic and hydrophobic properties. We previously reported the synthesis and characterization of polyamides and alternating poly(ester–amide)s obtained from aliphatic diamines containing enzymatically cleavable amide bonds [14,15], and of poly(ether–amide)s containing short oxyethylene sequences and enzymatically cleavable amide bonds [16]. This paper describes the preparation and the characterization of a set of hydrophilic aliphatic block poly(ether–ester–amide)s based on PLLA blocks, tri- and penta-ethylenoxide segments and α-aminoacids residues, which can be used in controlled drug release by oral administration. Accordingly, their biocompatibility has been tested using a cell line which mimics the intestinal epithelium.

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2. Experimental

2.1. Materials and techniques

The solvents were purified according to standard procedures. Tetraethyleneglycol ditosylate, Z-glycine (Z-Gly), Z-L-phenylalanine (Z-Phe), isobutylchloroformate (i-BCCl), tetrabutylammonium hydrogen sulphate (TBHS) (Fluka), Z-L-phenylalanylvaline (Z-Phe-Val) (Bachem), triethylamine and stannous 2-ethylhexanoate (Aldrich) were used as received. Sebacoyl dichloride and 4,7,10-trioxa-1,13-tridecanediamine (**1**) (Fluka) were purified by fractional distillation under vacuum. 1,12-Diaminododecane (DDA) (Fluka) was crystallized from anhydrous toluene. Poly(ethylene glycol) (PEG; $M_n = 400$) (Fluka) was dried by azeotropically removing the water with benzene using a Dean-Stark trap. L,L-Lactide (LA) (Polysciences) was crystallized from anhydrous ethyl acetate and dried in vacuo over P_4O_{10}. 1,4-Butanediol (BDL) (Fluka) was dried over calcium chloride and distilled before use. Dulbecco's modified Eagle's medium (DMEM) with 4.5 mg/ml glucose, fetal calf serum (FCS), non-essential amino acids, *N*-2-hydroxy-ethylpiperazine-*N*′-2-ethane sulfonic acid (HEPES), glutamine, penicillin, streptomycin, trypsin and PBS tablets were purchased from Gibco Life Science Technology. Trypan blue was obtained from Sigma Chemical.

Fourier transform infrared analysis was performed using a Jasco FTi.r.-430 spectrophotometer. The ^{1}H n.m.r. spectra were recorded at 25°C using a Varian Gemini instrument operating at 200 MHz and chloroform-d ($CDCl_3$) as solvent. The thermal data were obtained using a Perkin Elmer 7 differential scanning calorimeter (d.s.c.). Dynamic mechanical thermal analysis (d.m.t.a.) was performed using a Polymer Laboratories MK III analyzer on 0.4–0.5-mm thick films obtained by compression moulding. The purity of the products was checked by analytical RP-HPLC on a Varian 3000 LC Star System equipped with a 9065 Polycrome and a 9095 auto sampler. Wide-angle X-ray (WAXS) diffraction patterns were recorded at room temperature on a Philips PW 1711 diffractometer equipped with a continuous scan attachment and a proportional counter using Ni-filtered Cu Kα radiation (1.5418 Å). Transmission electron microscopy (t.e.m.) was performed by means of a TEM Philips EM 300 apparatus operating at 80 kV in a magnification range of 7000–30 000. Copolymer films were evaporated onto copper grids (100 mesh) from a very diluted (0.0005 g/dl) chloroform solution. Slow evaporation allowed reproducible morphologies to develop. Inherent viscosity was measured on a Cannon-Ubbelhode viscosimeter at 25°C in $CHCl_3$ ($c = 0.5$ g/dl). Average molecular weights were determined in tetrahydrofuran (THF) by size exclusion chromatography (s.e.c.) using a Waters 610 instrument fitted with a set of five columns and a refractive index detector; calibration was carried out with monodisperse polystyrene standards. Water absorption was determined at room temperature by dipping in liquid water, for 3 days, weighted polymer films (60–80 mg) prepared by compression moulding. Upon removal, the samples were blotted on filter paper to remove the excess water from the surface and weighted.

2.2. Cell culture

Human colon carcinoma cell lines (Caco-2) were routinely grown in 100-mm plastic dishes at 37°C in a humidified incubator with 5% CO_2/95% air atmosphere in a culture medium composed of DMEM supplemented with 10% FCS, glutamine (2 mM), penicillin (50 U/ml), streptomycin (50 U/ml), 1% non-essential amino acids and buffered with HEPES (25 mM) [17]. For daily experiments, the cells were detached from stock cultures by trypsinization and washed once by centrifugation before being seeded at a density of 10^5 cells/cm^2 in 6 well plates. Cultures were examined under an inverted light microscope on a regular basis in order to monitor growth and contamination.

2.3. Cytotoxicity assays

To evaluate the possible release of toxic substances by polymers, the cell culture medium was incubated for 24 h in the presence of a PEEA film. This medium was then moved to a well plate containing a confluent monolayer of Caco-2 cells. After 24 h, the cells were trypsinized, counted and tested for cell viability by trypan blue uptake [18]. In order to study the direct effect of PEEA on cell viability, glass dishes were coated with four samples of polymer and a small area was left uncovered in order to directly observe the cells by an inverted microscope. Before plating the Caco-2 cells, the PEEA films in the wells were left under UVC-irradiation for 12 h and then incubated for 18 h in the presence of an antibiotic solution, containing penicillin (10 000 U/ml) and streptomycin (10 000 μg/ml). 3×10^4 Caco-2 cells were seeded *per* each well. Six days after cell plating, the cells were observed and tested for viability.

2.4. Synthesis of diamines and amide–diamines

The 3,6,9,12,15-pentoxa-1,17-heptadecanediamine (**2**) was synthesized following the procedure reported by Kern et al. [19]. 1,13-Di(L-phenylalanylglycinamido)-4,7,10-trioxatridecane (**1a**) was prepared and characterized as described in a previous paper [16].

1,13-Di(L-phenylalanylvalylglycinamido)-4,7,10-trioxatridecane (**1b**) was directly prepared by reacting 1,13-di(glycinamido)-4,7,10-trioxatridecane with Z-Phe-Val, in the presence of i-BCCl and triethylamine, and by removing the Z group by hydrogenolysis on Pd/C catalyst.

Preparation of 1,17-di(L-phenylalanylglycinamido)-3,6,9,12,15-pentoxaheptadecane (**2a**): i-BCCl (2.30 ml; 17.1 mmol) was added dropwise at −15°C in a nitrogen atmosphere, under stirring, to 45 ml of a chloroform solution containing Z-Gly (3.30 g; 15.8 mmol) and triethylamine (2.40 ml; 17.1 mmol) contained in a two-necked

Table 1
Inherent viscosity, solubility and water absorption on PEEAs

Polymer code	Polyester	Diamine	η_{inh}[a] (g/dL)	Water absorption % H_2O	Solubility[b]				
					THF	Dioxane	*m*-Cresol	$CHCl_3$	Acetone
PEEA I	PLLA	1	0.53	13.0	+	+−	+−	++	++
PEEA II	PLLA	2	0.34	14.7	+	+	+−	++	++
PEEA Ia	PLLA	1a	0.47	13.4	+	+−	+	++	+−
PEEA IIa	PLLA	2a	0.33	21.0	+−	−	−	++	−
PEEA Ib	PLLA	1b	0.46	12.5	+	+−	+−	++	−
PEEA III	Triblock	1	0.55	3.2	+−	+−	+−	++	+−
PEEA IV	Triblock	DDA	0.53	2.1	+−	+−	++	++	−

[a] Inherent viscosity at 25°C in $CHCl_3$ ($c = 0.5$ g/dL).
[b] ++ very soluble; + soluble; +− sparingly soluble; − insoluble.

flask equipped with an inlet for nitrogen and a dropping funnel. After stirring for 20 min, a solution of **2** (2.00 g; 7.10 mmol) in 18 ml of chloroform was added dropwise at −15°C, and the mixture was stirred overnight at room temperature. The solution was washed with citric acid and $NaHCO_3$ solutions, water and finally dried over anhydrous Na_2SO_4. By removing the solvent at reduced pressure, 1,17-di(Z-glycinamido)-3,6,9,12,15-pentoxaheptadecane (**I**) was recovered as a yellow waxy solid (3.80 g; 5.7 mmol) which was washed with ethyl ether giving 3.2 g of a white solid (68% yield). **I** (3.0 g; 4.5 mmol) dissolved in methanol (180 ml) was treated with hydrogen in the presence of 10% Pd/C (0.48 g) yielding 1.5 g of 1,17-di(glycinamido)-3,6,9,12,15 pentoxaheptadecane (**II**) as a viscous colourless oil (85% yield). ^{1}H n.m.r.: $\delta = 3.35$ (4H; s); 3.48 (4H; m); 3.56 (4H; m); 3.65 (16H; d); 7,62 (2H; s). **2a** was obtained from **II** following a similar procedure: i-BCCl (1.10 ml; 8.5 mmol) was reacted with Z-L-Phe (2.40 g; 8.0 mmol) and triethylamine (1.20 ml; 8.5 mmol) dissolved in 23 ml of chloroform. Successively, 9 ml of chloroform containing **II** (1.50 g; 3.8 mmol) were added dropwise at −15°C to the reaction mixture. 1,17-Di(Z-L-phenylalanylglycinamido)-3,6,9,12,15-pentoxaheptadecane (**III**) (2.7 g; 74% yield) was recovered as a white solid (purity 97%). Hydrogenolysis of **III** gave 1.6 g of **2a** (85% yield) as white wax. ^{1}H n.m.r.: $\delta = 1.84$ (NH_2; s); 2.70, 3.28 (–CH_2CH–ϕ; 2m); 3,45 (–OCH_2CH_2-NH–CO–; t); ~3.5 (–CHCH_2–ϕ; m); 3.62–3.68 (–CH_2–O–; s); 3.98 (–NH–CH_2–CO–; d); 7.2–7.4 (–ϕ–; m); 7.46, 7.98 (–NH–CO–; t). FTi.r. (KBr): 3302 (ν_{NH}, amide), 3063 (ν_{CH}, aromatic), 1655 ($\nu_{C=O}$, amide), 1102 (ν_{C-O}, ether) cm^{-1}. Anal. Calc. for $C_{34}H_{52}N_6O_9$: C, 59.29; H, 7.61; N, 12.20; O, 20.90. Found: C, 58.84; H, 7.82; N, 12.11.

2.5. Synthesis of PLLA based macromers

Preparation of a α,ω*-hydroxyl terminated PLLA oligomer* (OH–PLLA–OH) ($M_n \cong 2000$): L,L-lactide (28.10 g; 195 mmol), 1,4-butanediol (1.30 g; 14.8 mmol) and stannous 2-ethylhexanoate (0.045 g) were reacted at 140°C for 24 h in a vacuum sealed glass vial. After cooling to room temperature, the vial was opened and the reaction product was recovered by dissolution in dichloromethane. After removing the solvent, the residue was heated to 100°C and 0.1 mmHg (29.10 g; 98% yield). Molecular weight: theor. = 2000; calcd. by ^{1}H n.m.r. ≅ 1900. ^{1}H n.m.r.: $\delta =$ 1.5 (–CH(CH_3)–OH, d); 1.58 (–CO–CH(CH_3)–O–, d); 1.7 (–CH_2CH$_2$O–, broad); 4.16 (–CH$_2$CH_2O–, broad); 4.36 (–CO–CH(CH$_3$)–OH; q); 5.18 (–CO–CH(CH$_3$)–O–; q); d.s.c.: $T_g = 50$°C, $T_m = 115$–125°C, $\Delta H_m = 12.8$ J/g.

Preparation of a α,ω*-hydroxyl terminated triblock PLLA–PEG–PLLA* (*OH–Triblock–OH*) ($M_n \cong 3300$) : LA (17.20 g; 119 mmol), PEG 400 (2.30 g; 5.7 mmol) and stannous 2-ethylhexanoate (0.0274 g) were reacted at 140°C for 24 h in a vacuum sealed glass vial. After cooling to room temperature, a treatment analogous to that followed for the PLLA oligomer gave 16.50 g of product (85% yield). Molecular weight: theor. = 3400; calcd. by ^{1}H n.m.r. ≅ 3300. ^{1}H n.m.r.: $\delta = 1.60$ (–CO–CH(CH_3)–CO–; d); 3.65 (–CH$_2$O–; s); 3.69 (–CH_2CH$_2$O–CO–; t); 4.28 (–CH_2O–CO–; t); 4.35 (–CO–CH(CH$_3$)–OH; q); 5.15 (–CO–CH–(CH$_3$)–O–; q); d.s.c.: $T_m = 141$°C, $\Delta H_m = 43.1$ J/g (d.s.c.).

2.6. Polymerization

The preparation of PEEA II (see Table 1) is reported as a typical procedure:

(a) preparation of a CO–Cl end-capped HO–PLLA–OH oligomer: a solution of the HO–PLLA–OH oligomer (3.40 g, 1.8 mmol) in 5.40 ml in anhydrous chloroform was added dropwise at $T = 50$°C under stirring to 0.851 g (3.6 mmol) of freshly distilled sebacoyl dichloride dissolved in 1.2 ml of an anhydrous chloroform/*n*-hexane mixture (1:1 by vol). After 15 min, 5.4 ml of anhydrous *n*-hexane were added and the solution was kept at this temperature for 2 h;
(b) interfacial polycondensation: the chloroform/*n*-hexane solution containing the CO–Cl end capped HO–PLLA–OH oligomers was quickly poured under vigorous stirring into a blender containing 80 ml of a water solution of **2** (0.507 g; 1.8 mmol) and KOH

(0.40 g) at 0–5°C. After 8 min, the resulting white polymer was collected on a glass filter, washed repeatedly with distilled water and dried in vacuo at 40°C giving 3.5 g of PEEA II (75% yield). In the case of PEEA IV (see Table 1), TBHS was added as emulsifying agent to the water solution before adding a chloroform solution of DDA.

3. Results and discussion

3.1. Synthesis and characterization of monomers

The diamines and amide–diamines used to prepare the PEEAs are shown in Fig. 1. Diamines **1** and **2** contain three and five hydrophilic ethylenoxide groups, respectively, whereas amide-diamines **1a**, **1b** and **2a** also contain two or three peptide bonds, one of which, generated by Phe, is susceptible to cleavage by α-chymotrypsine [9,20]. These amide–diamines were synthesized by sequential addition of aminoacid residues of Gly and Phe or of the Val-Phe dipeptide to diamines **1** or **2**, following a procedure reported in a previous paper [13]. The i.r. and ^{1}H n.m.r. spectra confirmed the structures expected. A α,ω-hydroxyl-terminated poly(L-lactide) (OH–PLLA–OH) and a α,ω-hydroxyl terminated triblock PLLA–PEG–PLLA (OH–Triblock–OH) macromers were synthesized by reacting in bulk appropriate amounts of LA using BDL [21] or PEG-400 [22] as initiators, respectively, and stannous 2-ethylhexanoate as a transesterification catalyst. The number average molecular weights, determined by ^{1}H n.m.r. spectroscopy by comparing the integral intensities of methyne resonances (5.15–5.18 δ) with those of $-CH_2O-$ resonances of BDL (4.16 δ) or PEG-400 (3.65 δ) were ≅ 1900 for OH–PLLA–OH and ≅ 3300 for the OH–Triblock–OH macromer. The structure of the latter copolymer may be ideally represented by a central PEG block ($M_n = 400$) having two PLLA arms ($M_n = 1450$). The wide-angle X-ray diffraction spectra of the macromers exihibit similar crystalline patterns, characterized by the presence of two strong reflexions at 2Θ angles of 16.7 and 19.2°. These values closely correspond to those found for high molecular weight PLLA (16.2 and 18.6°), indicating the development of a crystalline structure of the PLLA type. Accordingly, both macromers show melting peaks in the d.s.c. thermograms. The melting temperatures, 115–125°C for OH–PLLA–OH and 141°C for OH–Triblock–OH, are much lower than that of high MW PLLA, 175°C, in agreement with the low MW of the PLLA segments in the macromers. It is worth noting that the melting of the PLLA blocks in the triblock macromer occurs at a temperature higher than that of the corresponding blocks in OH–PLLA–OH, thus indicating that the presence of the short and flexible PEG central segment in OH–Triblock–OH favours the arrangement of the PLLA segments in more regular crystals as suggested also by the enhancement of the melting enthalpy (43.1 and 12.8 J/g for OH–Triblock–OH and OH–PLLA–OH, respectively). Such effect was not reported in literature for PLLA/PEG/PLLA copolymers with long PEG segments [22,23].

3.2. Synthesis and characterization of polymers

PEEAs with different chain flexibility, hydrophilicity, and number of aminoacid residues (0–3) were prepared from the HO–PLLA–OH or HO–Triblock–OH macromers, sebacoyl chloride (Seb) and diamines **1**,**2** or amido-diamines **1a**, **2a**, **1b** following the "two-step route" outlined in Fig. 2. The stoichiometric molar ratio macromer/Seb/Diamine = 1:2:1 was used in this procedure. In the first step, the macromer was reacted with a two-fold excess of sebacoyl chloride to obtain a sebacoyl-COCl end-capped intermediate (**I**). In the second step, the chain extension with a diamine (or an amido-diamine) was performed following the stirred interfacial polycondensation technique by dissolving the diamine and an acid acceptor in water. In the case of DDA, which is insoluble in water, a concentrated $CHCl_3$ solution of diamine was emulsified, under vigorous stirring, with the appropriate volume of water containing the acid acceptor and TBHS as emulsifying agent. The copolymer's architecture is of the multiblock type with a well defined length of the etheramide segments. The PEEAs were obtained in high yields (70–90%) as white powders which are readily soluble in chloroform, moderately soluble in THF and insoluble in water. Tough, flexible films were prepared by solution casting from chloroform or by melt casting. In Table 1, the inherent viscosity values of the prepared PEEA are reported, together with the results of solubility and water absorption tests. The inherent viscosity values are in the range 0.33–0.55 dl/g and are indicative of moderate polymerization degrees. Reliable M_n values of PEEA I, **Ia** and **IIa** (M_n = 23, 24 and 16 kDa, respectively) were obtained by s.e.c. Rather narrow molecular weights distributions curves were found, as indicated by polydispersity index values in the range 1.5–2.1. The molecular structure of prepared polymers was confirmed by i.r. and ^{1}H n.m.r. spectroscopy measurements. The i.r. spectra obtained on polymer films show characteristic amide bands at 1655–1650 cm^{-1} and 1545–1540 cm^{-1}, ester bands at 1760, 1185 and 1132 cm^{-1}, and absorption of the C–O–C ether group at 1092 cm^{-1}. The ^{1}H n.m.r. spectra of PEEAs exihibit all the expected resonance. The formation of a block copolymer strucrure was confirmed by the presence of both CH_2–CO–NH and CH_2–COO– resonances of comparable intensity at 3.3–3.4 δ and at 2.1–2.2 δ, respectively. The compositions were determined by comparing the integral intensities of methyne hydrogens of PLLA blocks at 5.1–5.2 δ with those of $-CH_2O-$ hydrogens of oxyethylene sequences at 3.6–3.7 δ.

The hydrophilicity of the polymers prepared was investigated by determining the equilibrium concentration of absorbed liquid water at room temperature. The PEEAs **I**, **Ia** and **Ib** show high and comparable weight uptake after immersion for 3 days in water (≈12.5–13.4% by wt). The

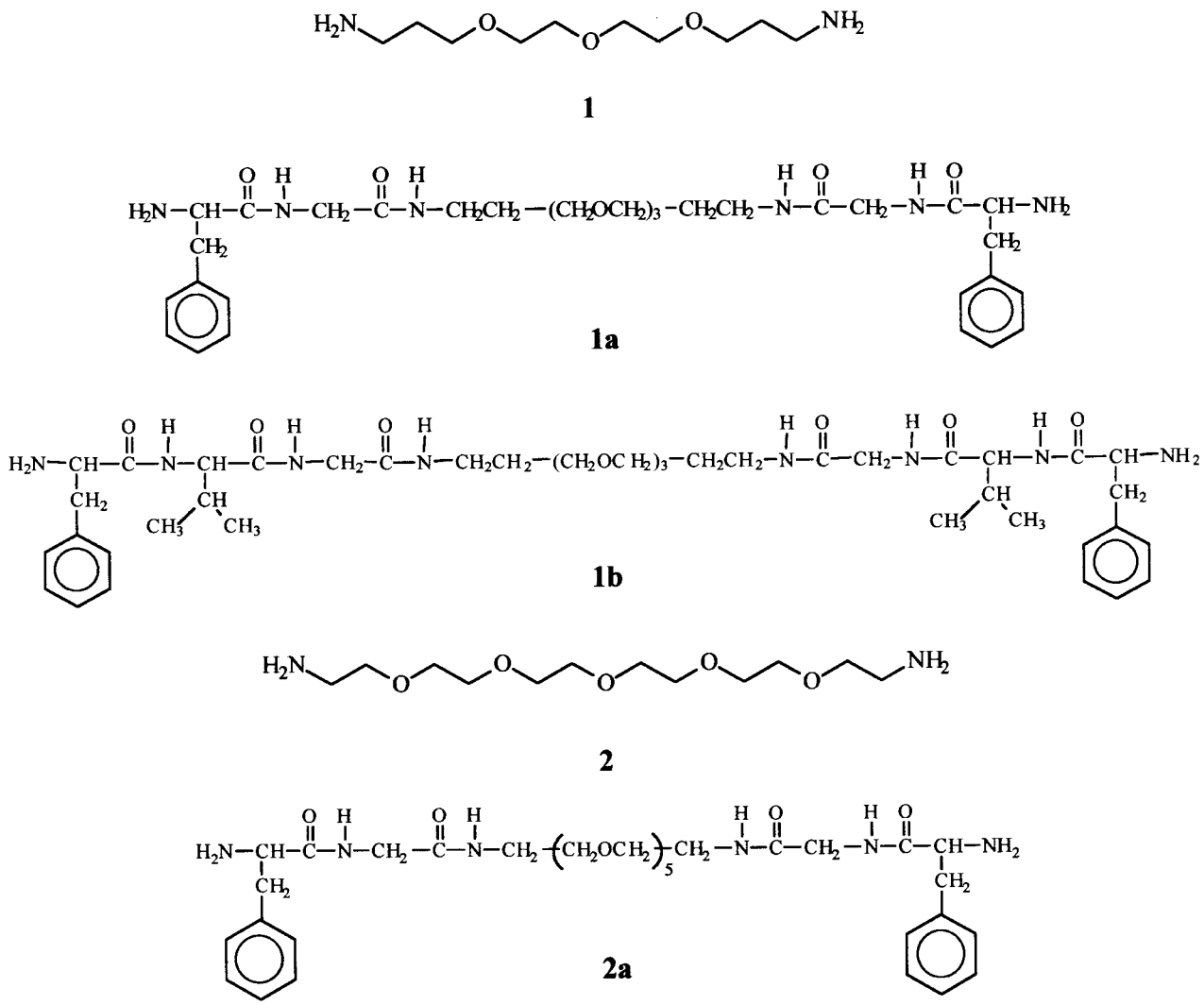

Fig. 1. Structure of diamines and amide-diamines used to prepare the PEEAs.

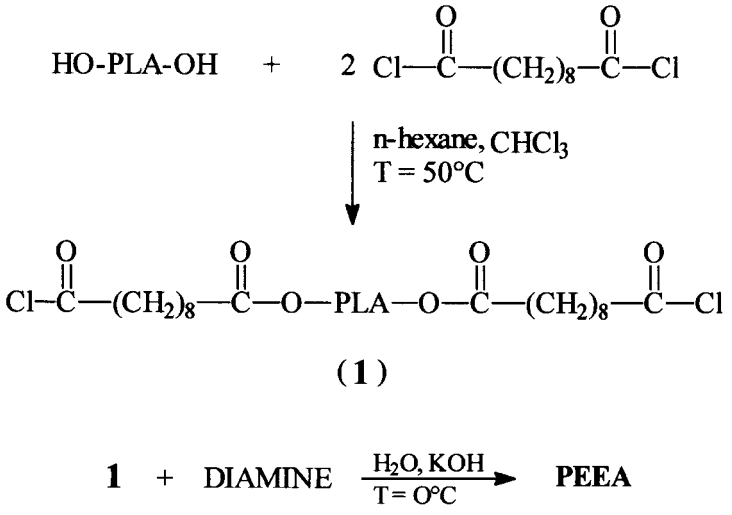

Fig. 2. Scheme of the two-step polymerization method used to prepare segmented PEEAs.

Table 2
Thermal properties and crystallinity degree of PEEAs

Polymer	T_m [a] (°C)	T_g [b] (°C)	T_g [c] (°C)	ΔH_m [a] J/g	X_c [d] (%)
PEEA I	112	32	37	7.7	27
PEEA II	111	40	40	8.5	32
PEEA Ia	112	40	45	6.5	29
PEEA IIa	100–120	41	45	8.9	31
PEEA Ib	94; 110	38	42	5.0	31
PEEA III	135 80[e]	31	35	32.0	37
PEEA IV	131 62[e]	53	38	26.5	43

[a] Melting temperature, T_m, and apparent enthalpy, ΔH_m, measured by d.s.c.
[b] Glass transition temperature, T_g, measured by d.s.c.
[c] T_g measured by d.m.t.a.
[d] Degree of crystallinity obtained from X-rays diffraction patterns.
[e] Temperature of cold crystallization.

presence of α-aminoacids residues, as well as their number, does not affect the content of absorbed water. In fact, PEEA Ia and PEEA Ib, which contain two dipeptide or tripeptide residues for chain repeat unit, absorb amounts of water comparable to that of PEEA I. The length of the oxyethylene sequences does not significantly influence the hydrophilicity of PEEAs when the aminoacid residues are absent (compare PEEA I and PEEA II), while a more noticeable effect is observed in the presence of dipeptide units (compare PEEA Ia and PEEA IIa). PEEAs III and IV, which contain PLLA–PEG–PLLA blocks, show a small water uptake (3.2 and 2.1%, respectively). This finding is not surprising in the case of PEEA IV considering the presence of the hydrophobic DDA diamine and, moreover, it indicates that the presence of the PEO 400 central segment in the PLLA block does not increase PEEA hydrophilicity, probably due to "structural hindrance". In fact, we hypothize that the short amorphous PEO segments are entrapped within the PLLA crystalline structure where the water molecules cannot penetrate. Nevertheless, the amount of water absorbed by PEEA III is very small if compared with other PEEAs derived from tri(oxyethylene) based diamines. It is reasonable to assume that this effect depends on the structural organization of the polymer chains and, in particular, may be related to the high crystallinity of PEEA III (vide infra).

3.3. Thermal properties and morphology of polymers

Thermal behaviour was examined by d.s.c and d.m.t.a. and the relevant data are reported in Table 2. The d.s.c. thermograms of PEEA I and PEEA III are presented in Fig. 3 as representative examples of the polymers prepared. All the PEEAs prepared are semicrystalline, as shown by the presence of melting endotherms in their d.s.c. traces. PEEA I samples, both as obtained from the synthesis or as thin films obtained by solution casting, exhibit a broad melting peak with a maximum at $T \cong 112$°C, which disappears in a second heating run after rapid cooling (curves a and b). The melting temperature is very close to that of the parent HO–PLLA–OH macromer. Fairly similar behaviour was observed for other HO–PLLA–OH based polymers. The T_m and ΔH_m values in this class of PEEAs ranged between 94 and 120°C and 5.0–8.9 J/g. Their WAXS showed crystalline patterns analogous to that of PLLA with two strong diffraction peaks at $\cong 16.2$ and $\cong 18.6°$ of 2Θ [24]. Thus, we may conclude that these polymers show a PLLA-type crystalline organization and have a low tendency to crystallize from the melt. In the case of PEEAs derived from the triblock macromer, the first heating run (curve c of Fig. 3) shows a sharp endotherm at 135°C which closely corresponds to that found for the parent macromer (141°C). After a rapid cooling, the second heating run (curve d of Fig. 3) reveals a cold crystallization process at $\cong 80$°C followed by the melting at 133°C. In both runs the melting

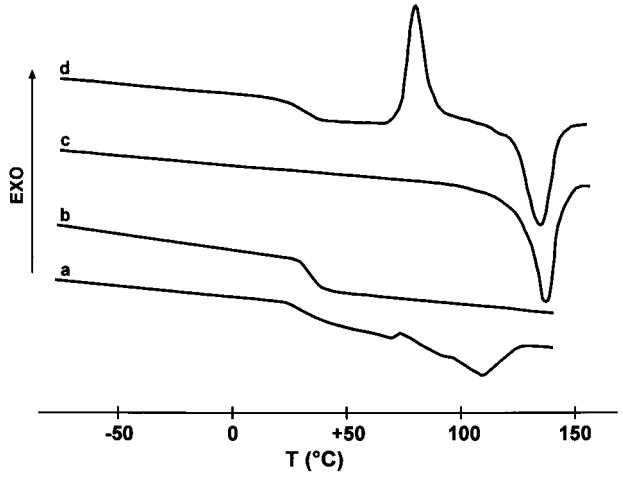

Fig. 3. D.s.c. traces of PEEA I (a = first heating run; b = second heating run) and PEEA III (c = first heating run; d = second heating run).

Table 3
Polymer effects on Caco-2 cell growth and cell viability

	n[a]	Cell number[b] ($\times 10^6$)	Cell viability (%)
Control medium	4	1.01 ± 0.07	100
PEEA Ia medium	4	1.06 ± 0.10	100
PEEA IIa medium	4	1.17 ± 0.04	99
Without film	4	0.69 ± 0.05	100
PEEA Ia film	3	0.64 ± 0.09	100
PEEA IIa film	3	0.65 ± 0.05	100

[a] Number of specimens tested.
[b] Results are expressed as mean ± SE.

enthalpies of PEEAs III and IV (25.4 and 32.0 J/g, respectively) are much higher than those found in the case of other PEEAs and their high crystallinity (≥37%) is confirmed by WAXS. Thus, the positive influence of the PEG segments on the the PLLA blocks ability to crystallize, which was already found in the macromer precursor (see Section 3.1), is preserved in the polymer, likely because the PEG central flexible blocks would provide increased conformational freedom to allow the PLLA "arms" to crystallize more easily. The increased crystallinity of PEEAs III and IV together with the high content of the hydrophobic polyester component, may account for their low water absorption values, suggesting that the hydrophilic PEG blocks can be — at least partially — involved in the PLLA crystalline structure.

The glass transition temperatures can be detected in second d.s.c runs. Second order transitions appear, in fact, within the 31–41°C temperature range. The d.m.t.a. spectra of melt cast PEEAs, performed from −100°C to 80°C confirm the presence of a main relaxation in the above temperature range, as evidenced by a band of the loss factor tanδ and a simultaneous decrease of the elastic modulus. The d.m.t.a. spectrum of PEEA IIa is reported in Fig. 4 as an example. The T_g determined by the two techniques, reported in Table 2, are in fair agreement and are scarcely dependent on the chemical structure of the polymer. Considering that the polyester component accounts for the 60–70 wt% of the polymers, it is reasonable to assume that this transition is related to the amorphous PLLA phase. The T_g values are, however, significantly lower than that of high MW PLLA (78°C) and this lowering may be due to the low MW of PLLA segments. Moreover, the PLLA amorphous phase may also include the SEB residues and possibly some ether-amide (or PEG) segments. Clear evidence of a glass transition related to the latter segments was not found in the d.s.c. thermograms. A low-intensity relaxation band in the low temperature range was found in the d.m.t.a. spectra of amino-acid containing PEEAs (see Fig. 4). The intensity of the latter band is, however, too low to be considered as an indication of microphase separation. Therefore, the PEEAs were also investigated by t.e.m., a technique frequently employed to study the microstructural organization of block copolymers.

It is well established in literature that when the blocks of a copolymer are built by similar elements or similar chemical bonds, it is quite difficult to gain intrinsic electronic contrast between different blocks and staining techniques are required. In the present case, direct casting of PEEA films from a very diluted (0.5 mg/ml) chloroform solution onto copper grids for t.e.m. analysis was attempted. Films were efficiently deposited and analysed in natural electronic contrast. Fig. 5 shows the micrographs of the aminoacid containing PEEA Ia and IIa at the same magnification. The existence of a superstructure in these copolymers, which is made by darker tiny walls surrounding clear larger

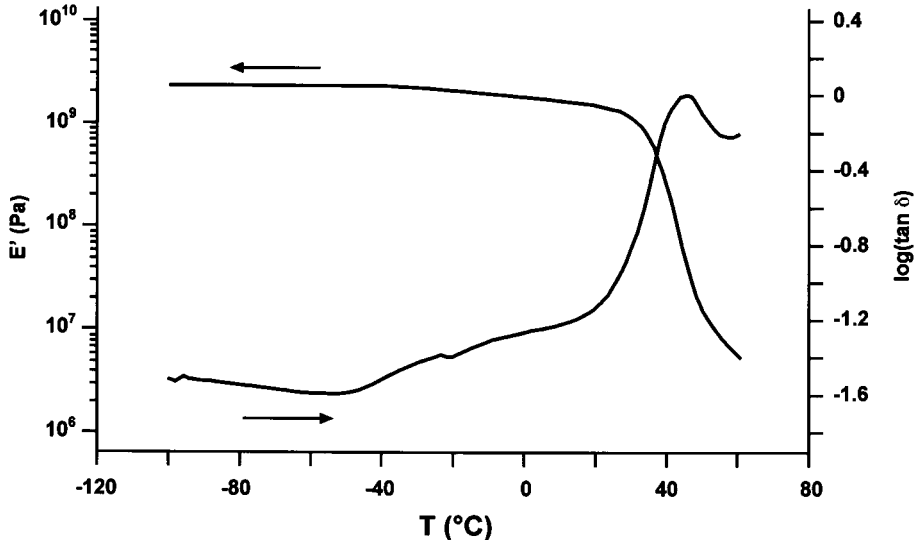

Fig. 4. Dynamical mechanical thermal analysis (d.m.t.a.) spectrum of PEEA IIa (E' = elastic modulus; tan δ = loss factor).

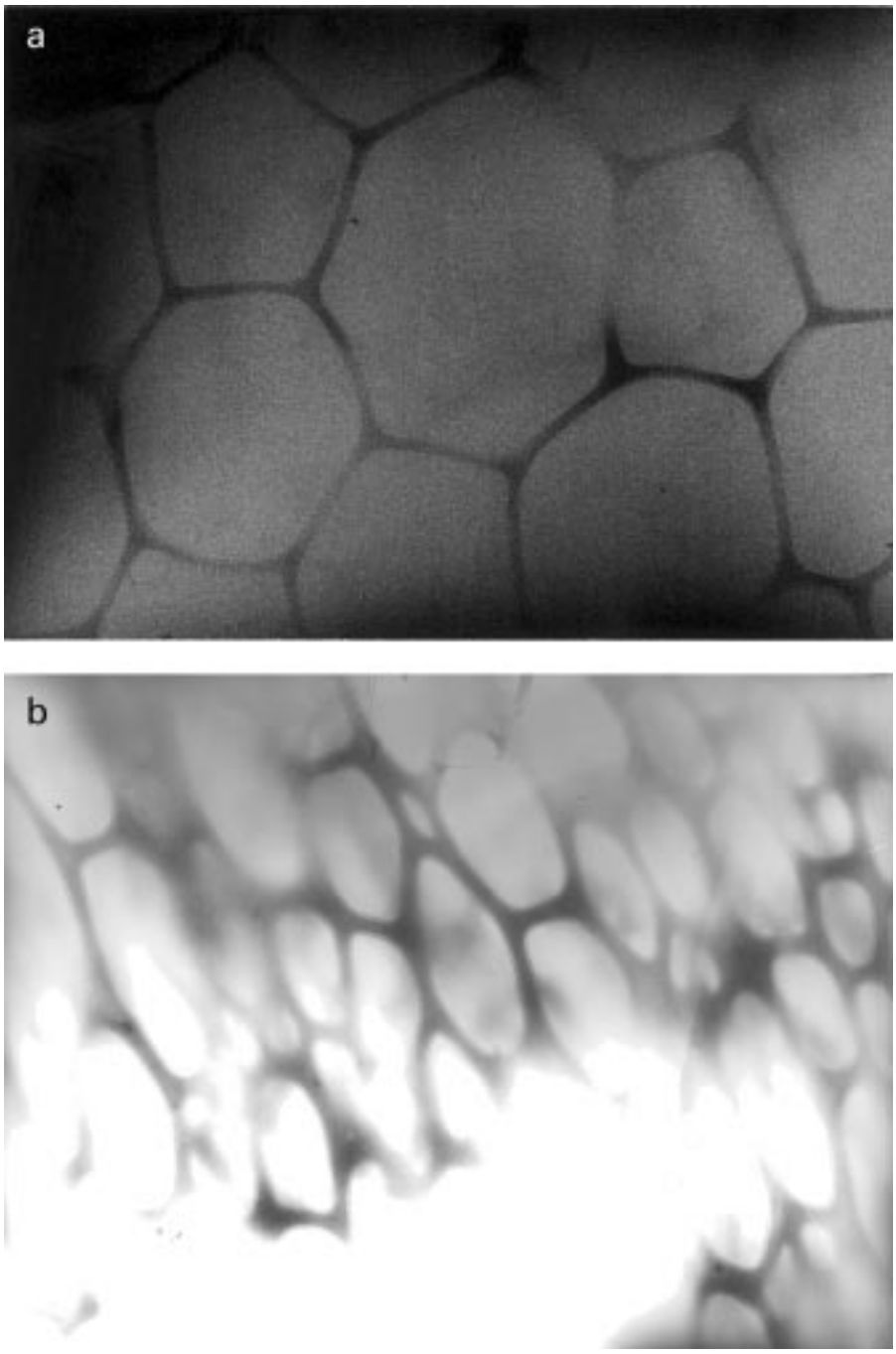

Fig. 5. T.e.m. pictures of (a) PEEA Ia and (b) PEEA IIa. Marker size = 200 nm.

domains of 0.1–0.5 μm size, is clearly observable. It is supposed that the darker regions are due to hydrogen bonded aminoacids containing segments with high electron density phenyl rings, while the clear areas are due to the polyester component. Although such kind of microphase organization is not frequently encountered in literature, fairly similar structures were nevertheless reported for urethane based block copolymers [25]. A comparison of the average thickness of the dark walls (~20 and ~10 nm for PEEA IIa and PEEA Ia, respectively) suggests that the size of the superstructural elements may be related to the length of the etheramide segments. No phase contrast was detected in the case of aminoacid-free PEEAs, and so far it is not known whether this is due to the absence of a superstructural organization or to the lack of the necessary electronic contrast when the phenyl-containing aminoacids are absent. Unfortunately, no efficient staining technique is at present available for the latter polymers.

Fig. 6. Phase contrast microscopic appearance of Caco-2 cell monolayer grown on PEEA IIa polymer. The cells are tightly packed on the polymer film (black area).

3.4. Cell viability

Intestinal cells play a fundamental role as barrier between potentially dangerous substances ingested and internal organs. Therefore, the Caco-2 cell line was selected as model system in order to evaluate the biocompatibility of thin films of two aminoacid-containing polymers (PEEA Ia and PEEA IIa). Indeed, these cells, originally derived from a human colon carcinoma, when grown in culture undergo enterocytic differentiation and form a polarized monolayer closely resembling, both morphologically and functionally, the small intestinal epithelium [26]. In this respect, this cell line was widely used for the study of a variety of intestinal functions, including the interaction between the polarized epithelium and exogenous molecules [27,28].

To investigate whether polymers release toxic agents, the cell culture medium was first incubated with the film being tested, and then placed on Caco-2 cells. After 24 h, cell growth and viability were tested by counting cell numbers and by trypan blue uptake. The results reported in Table 3 indicate that both PEEA Ia and PEEA IIa films do not release compounds, which are toxic for Caco-2 cells. Polymer biocompatibility was then investigated by employing an in vitro model according to the direct contact method in which the cells are directly cultured on the polymer films. Six days after cell plating, the cell number and cell viability were evaluated. Regardless of the chemical structure, no difference in reaching confluence (see Fig. 6) and cell viability (Table 3) was observed in the cells grown on PEEAs films compared with control. In conclusion, these data suggest that the investigated polymers should not exert toxic effects at intestinal level.

4. Conclusions

Biocompatible segmented PEEAs with a wide range of properties can be prepared from SEB, PLLA-based macromers and diamines containing hydrophilic short ethyleneoxy sequences either alone or linked with di- or tripeptide sequences. The bulk properties of PEEAs, such as T_m, ΔH_m, T_g, and water absorption mainly depend on the structure of the PLLA macromer. Evidence of microphase separation was obtained by t.e.m. in the case of aminoacids containing PEEAs. The biodegradability of these copolymers, characterized by the presence of both hydrolytically degradable ester groups and enzymatically degradable peptide bonds, and the possibility to prepare drug delivery microspheres are currently under investigation.

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